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**THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants : Fergal Conan Hill, et al.  
U.S. Serial No. : 10/007,628  
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For : PROTEIN SCAFFOLD AND ITS USE TO MULTIMRISE  
MONOMERIC POLYPEPTIDES

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New York, NY 10151

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February 21, 2002

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**CLAIM OF PRIORITY**

Assistant Commissioner for Patents  
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Sir:

Applicants hereby claim priority under 35 U.S.C. §119 and/or 120, from British  
application number GB9928831.8, GB9928788.0, GB9911298.9 and International patent  
application number PCT/GB00/01815, a certified copy of each is enclosed.

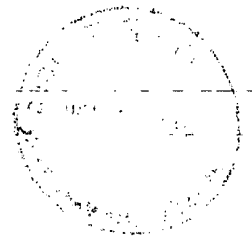
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Respectfully submitted,

FROMMER LAWRENCE & HAUG LLP

By:

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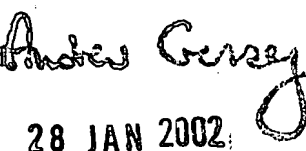
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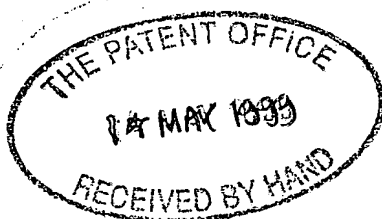
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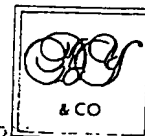


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4. Title of the invention

Oligomeric Proteins

5. Name of your agent (if you have one)

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01703 634816

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## Oligomeric Proteins

The present invention relates to oligomers of polypeptides or polypeptide domains. In particular, the invention relates to polypeptides constructed by oligomerisation of minichaperone monomers to form a heptameric ring structure.

Proteins, especially catalytic proteins (enzymes) and proteins which have biological activities, are dependent on tertiary structure for most or all of their functional attributes. Tertiary structure, which is defined by the three-dimensional arrangement of the protein, is dependent on the folding of the primary polypeptide sequence in three dimensions. The tertiary structure is stabilised by the interaction between parts of the primary sequence in the folded state, such as the formation of disulphide bonds, and energetic considerations deriving from juxtaposition of particular chemical entities in the three-dimensional arrangement.

It is known that the tertiary structure of proteins, particularly proteins which are stored for any length of time, can degrade, leading to suboptimal activity. This can be due to a variety of factors, including aggregation and the formation of improper intra- and inter-molecular interactions. Moreover, it is also known that proteins produced by recombinant DNA technology are frequently misfolded, especially if produced in bacterial expression systems. The strongly reducing environment present in bacterial cytoplasm impedes the formation of correct disulphide bonds, thus obstructing the folding process.

Many proteins require the assistance of molecular chaperones in order to be fold *in vivo* or to be refolded *in vitro* in high yields. Molecular chaperones are proteins, which are often large and require an energy source such as ATP to function. A key molecular chaperone in *Escherichia coli* is GroEL, which consists of 14 subunits each of some 57.5 kD molecular mass arranged in two seven membered rings. There is a large cavity in the GroEL ring system, and it is widely believed that the cavity is required for successful protein folding activity. For optimal activity, a co-chaperone, GroES, is required which consists of a seven membered ring of 10 kD subunits. The activity of the GroEL/GroES complex requires energy source ATP.

Some proteins are monomers, consisting of a single subunit. Many proteins are oligomeric, consisting of more than one subunit. Sometimes the subunits are identical, sometimes there are different types of subunits. Frequently, the subunits are linked non-covalently. Sometimes, the subunits are attached covalently, with a stretch of polypeptide linking the C-terminus of one domain to the N-terminus of another.

Allosteric proteins are a special class of oligomeric proteins, which alternate between two or more different three-dimensional structures on the binding of ligands and substrates. Allosteric proteins are often involved in control processes in biology or where mechanical and physico-chemical energies are interconverted.

GroEL is an allosteric protein. The role of ATP is to trigger this allosteric change, causing GroEL to convert from a state that binds denatured proteins tightly to one that binds denatured proteins weakly. The co-chaperone, GroES, aids in this process by favouring the weak-binding state. It may also act as a cap, sealing off the cavity of GroEL. Further, its binding to GroEL is likely directly to compete with the binding of denatured substrates. The net result is that the binding of GroES and ATP to GroEL which has a substrate bound in its denatured form is to release the denatured substrate either into the cavity or into solution where it can refold.

Minichaperones have been described in detail elsewhere (see International patent application WO99/05163, the disclosure of which is incorporated herein by reference). Minichaperone polypeptides possess chaperoning activity when in monomeric form and do not require energy in the form of ATP. Defined fragments of the apical domain of GroEL of approximately 143-186 amino acid residues in length have molecular chaperone activity towards proteins either in solution under monomeric conditions or when monodisperse and attached to a support.

The activity of minichaperones, although sufficient for many purposes, is inferior to that of intact GroEL. There is thus a need for a more active form of minichaperone, which nevertheless retains independence from energy requirements.



## Summary of the Invention

According to the present invention, there is provided a polypeptide oligomer comprising  
5 two or more monomers, each monomer comprising an amino acid sequence fused to a subunit of a heterologous oligomerisable scaffold.

The monomer of the invention is defined as such in that it is capable of associating with  
further monomers, whether homologous or heterologous, to form a multimeric (or  
10 oligomeric) protein comprising a plurality of monomers. The ability to oligomerise is a property of the oligomerisable scaffold, which is a protein structure which is capable of assembly into an oligomeric structure. This can be a dimeric, trimeric, tetrameric or higher-order structure. Advantageously, it is a heptameric structure.

15 As used herein, the terms "oligomer" and "multimer" are equivalent and imply no upper limit in the number of subunits present.

An "amino acid sequence", as referred to herein, is a polypeptide which is fused to the scaffold. Any polypeptide may be used, which is heterologous to the scaffold, and which  
20 it is desirable to oligomerise. Preferred polypeptides include minichaperone polypeptides, antigenic polypeptides, enzymes and any polypeptide which is capable of mediating a biological effect. Oligomerisation of such polypeptides allows their spatial juxtaposition, which may potentiate their activity. Moreover, if the oligomer is heterogeneous, oligomeric constructs according to the invention permit the juxtaposition of a plurality of  
25 biological activities which can be brought to bear on a single molecule contemporaneously.

Polypeptides are known in the art which are capable of oligomerising. Many such polypeptides are suitable to provide scaffolds for use in the present invention. For  
30 instance, the protein glutathione-S-transferase (GST) is capable of dimerising; moreover, it is commonly used as a fusion "tag" to isolate proteins from solutions. Use of GST as an oligomerisable scaffold thus allows the production of dimers. In a preferred embodiment,

the oligomerisable scaffold is the bacteriophage T4 gene product (Gp) 31, which forms a heptameric ring structure. Gp31 functionally substitutes for the bacterial co-chaperonin GroES for the GroEL-assisted protein reaction both *in vitro* and *in vivo* (Laemmli, U. K., *et al.*, (1970) *J. Mol. Biol.* **47**, 69-85; van der Vies, S., *et al.*, (1994) *Nature* **368**, 654-656; GenBank Accession No. AAA32510) and forms a stable structure of seven ~12 kDa subunits. The three-dimensional structure illustrates that a highly mobile loop projects from each subunit, between positions 27 and 42. The mobile loop is a preferred site for heterologous amino acid sequence insertion.

10 In a second aspect of the present invention, there is provided a polypeptide comprising two or more minichaperone proteins covalently linked together. Covalent linkage may be achieved, for example, by chemical cross-linking or by fusion using recombinant DNA technology.

15 The minichaperones are preferably arranged in a ring formation, which may comprise any number of members, for example between 2 and 20 members or more, preferably between 5 and 10 members, and most preferably about 7 members. Minichaperone polypeptides according to either the first or second aspects of the present invention are referred to herein as "minichaperone oligomers".

20

According to a third aspect of the present invention, there is provided a method for promoting the folding of a polypeptide comprising contacting the polypeptide with a multimeric minichaperone polypeptide as described above and a foldase.

25 The polypeptide which is folded by the method of the invention is preferably an unfolded or misfolded polypeptide, and advantageously comprises a disulphide. The minichaperone polypeptide preferably comprises fragments of a molecular chaperone, preferably fragments of any hsp-60 chaperone, and may be selected from the group consisting of mammalian hsp-60 and GroEL, or a derivative thereof.

30

In the case that the fragment is a fragment of GroEL, it advantageously does not have an Alanine residue at position 262 and/or an Isoleucine residue at position 267 of the

sequence of intact GroEL as defined at GenBank Accession No. P06159. Preferably, it has a Leucine residue at position 262 and/or a Methionine residue at position 267 of the sequence of intact GroEL. The invention therefore encompasses the use of a fragment of GroEL comprising a Leucine residue at position 262 and/or a Methionine residue at position 267 of the sequence of intact GroEL for promoting the folding of a polypeptide.

In a preferred embodiment, the minichaperone comprises a region which is homologous to at least one of fragments 193-335, 191-376, 191-345 and 191-335 of the sequence of intact GroEL.

Advantageously, the foldase is selected from the group consisting of thiol/disulphide oxidoreductases and peptidyl prolyl isomerases.

Preferably, the thiol/disulphide oxidoreductase is selected from the group consisting of *E. coli* DsbA and mammalian PDI, or a derivative thereof. Preferably, the peptidyl prolyl isomerase is a cyclophilin.

The invention moreover concerns a method as described above wherein the minichaperone oligomer and/or the foldase is immobilised onto a solid phase support, which may be agarose. Accordingly, the invention also provides a solid phase support having immobilised thereon a minichaperone oligomer and/or a foldase and a column packed at least in part with such a solid phase support.

In a further aspect, the present invention provides a composition comprising a combination of a minichaperone oligomer and a foldase, optionally together with a diluent, carrier or excipient.

#### Brief Description of the Figures

**Figure 1.** (a) Three-dimensional structure of Gp31 of bacteriophage T4 solved at 2.3 Å (27). Positions mentioned in the text are indicated (residues numbered as in (26)). (b) Three-dimensional structure of minichaperone GroEL(191-376) solved at 1.7 Å (22).

Positions mentioned in the text are indicated (residues numbered as in (2)). Secondary structure representations are drawn with MolScript (49).

**Figure 2.** Schematic representation of Gp31 proteins in the vectors used in this study.

5 The presence of the Gp31 mobile loop (residues 23 to 44) and/or minichaperone GroEL (residues 191 to 376) are indicated by boxes. The nucleotide sequence of the Gp31 mobile loop and relevant restriction sites are shown. The names of the corresponding vector are listed in the left margin.

10 **Figure 3.** Molecular weight determination by analytical gel filtration chromatography. Wild-type proteins Gp31 ( $M_r$  ~7 x 12 kDa) and GroEL(191-376) ( $M_r$  ~22 kDa) and, Gp31 $\Delta$ loop and Gp31 $\Delta$ ::GroEL(191-376) (MC<sub>7</sub>) were run on a Superdex™ 200 (HR 10/30) column (Pharmacia Biotech.) calibrated with molecular weight standards (solid-line and circles). Gp31 $\Delta$ loop and MC<sub>7</sub> eluted at volumes corresponding to molecular  
15 weights of ~145.6 and ~215 kDa, respectively.

**Figure 4.** Characterization of MC<sub>7</sub> by CD spectroscopy. (a) Far UV-CD spectrum at 25 °C. (b) Thermal denaturation followed at 222 nm at a heating rate of 1 °C.min<sup>-1</sup>.

20 **Figure 5.** (a) Binding specificity of MC<sub>7</sub> to GroES determined by ELISA. (b) Inhibition of MC<sub>7</sub> binding to heptameric co-chaperonin GroES by varying concentrations of synthetic peptide corresponding to residues 16 to 22 of GroES mobile loop determined by competition ELISA.

25

## Detailed Description of the Invention

### Definitions and detailed description

30 *Oligomerisable scaffold.* An oligomerisable scaffold, as referred to herein, is a polypeptide which is capable of oligomerising and to which a heterologous polypeptide

may be fused, preferably covalently, without abolishing the oligomerisation capabilities. Thus, it provides a "scaffold" using which polypeptides may be arranged into multimers in accordance with the present invention. Substantially any polypeptide capable of forming a multimer may be used as a scaffold. Optionally, parts of the wild-type polypeptide from which the scaffold is derived may be removed, for example by replacement with the heterologous polypeptide which is to be presented on the scaffold.

Any protein capable of forming an oligomer may be used as a scaffold. Preferred are polypeptides such as GST, which may be fused with desired heterologous polypeptides according to well established techniques.

In a preferred embodiment, the scaffold polypeptide is based on GroES or an analogue thereof. A highly preferred analogue is the T4 polypeptide Gp31. GroES analogues, including Gp31, possess a mobile loop (Hunt, J. F., *et al.*, (1997) *Cell* **90**, 361-371; Landry, S. J., *et al.*, (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11622-11627) which may be inserted into, or replaced, in order to fuse the heterologous polypeptide to the scaffold.

Gp31 with a deleted mobile loop possesses no biological activity, making it an advantageously inert scaffold, thus minimising any potentially deleterious effects. Insertion of an appropriate biologically active polypeptide, such as a minichaperone polypeptide, can confer a biological activity on the novel polypeptide thus generated. Indeed, the biological activity may be improved by incorporation of the biologically active polypeptide into the scaffold.

Alternative sites for peptide insertion are possible. An advantageous option is in the position equivalent to the roof beta hairpin in GroES. This involves replacement of Glu-60 in Gp31 by the desired peptide. The amino acid sequence is Pro(59)-Glu(60)-Gly(61). This is conveniently converted to a SmaI site at the DNA level (CCC:GGG) encoding Pro-Gly, leaving a blunt-ended restriction site for peptide insertion as a DNA fragment. Alternatively, inverse PCR may be used, to display the peptide on the opposite side of the scaffold.

*Oligomer.* As used herein, "oligomer" is synonymous with "polymer" or "multimer" and is used to indicate that the object in question is not monomeric. Thus, oligomeric polypeptides according to the invention comprise at least two monomeric units joined together covalently or non-covalently. The number of monomeric units employed will  
 5 depend on the intended use of the oligomer, and may be between 2 and 20 or more. Advantageously, it is between 5 and 10, and preferably about 7.

*Polypeptide.* As used herein, a polypeptide is a molecule comprising at least one peptide bond linking two amino acids. This term is synonymous with "protein" and "peptide",  
 10 both of which are used in the art to describe such molecules. A polypeptide may comprise other, non-amino acid components. The polypeptide the folding of which is promoted by the method of the invention may be any polypeptide. Preferably, however, it is an unfolded or misfolded polypeptide which is in need of folding. Alternatively, however, it may be a folded polypeptide which is to be maintained in a folded state (see below).  
 15

Preferably, the polypeptide contains at least one disulphide. Such polypeptides may be referred to herein as *disulphide-containing polypeptides*.

Examples of polypeptides include those used for medical or biotechnological use, such as  
 20 interleukins, interferons, antibodies and their fragments, insulin, transforming growth factor, and many toxins and proteases, as well as molecular chaperones and minichaperones, peptidyl-prolyl isomerases and thiol/disulphide oxidoreductases.

*Promoting the folding.* The invention envisages at least two situations. A first situation is  
 25 one in which the polypeptide to be folded is in an unfolded or misfolded state, or both. In this case, its correct folding is promoted by the method of the invention. A second situation is one in which the polypeptide is substantially already in its correctly folded state, that is all or most of it is folded correctly or nearly correctly. In this case, the method of the invention serves to maintain the folded state of the polypeptide by affecting  
 30 the folded/unfolded equilibrium so as to favour the folded state. This prevents loss of activity of an already substantially correctly folded polypeptide. These, and other, eventualities are covered by the reference to "promoting" the folding of the polypeptide.

*Contacting.* The reagents used in the method of the invention require physical contact with the polypeptides whose folding is to be promoted. This contact may occur in free solution, *in vitro* or *in vivo*, with one or more components of the reaction immobilised on solid supports. In a preferred aspect, the contact occurs with the minichaperone oligomer and/or the thiol/disulphide oxidoreductase immobilised on a solid support, for example on a column. Alternatively, the solid support may be in the form of beads or another matrix which may be added to a solution comprising a polypeptide whose folding is to be promoted.

10

*Fragment.* When applied to chaperone molecules, a fragment is anything other than the entire native molecular chaperone molecule which nevertheless retains chaperonin activity. Advantageously, a fragment of a chaperonin molecule remains monomeric in solution. Preferred fragments are described below. Advantageously, chaperone fragments are between 50 and 200 amino acids in length, preferably between 100 and 200 amino acids in length and most preferably about 150 amino acids in length. Fragments of chaperone molecules which remain monomeric in solution and possess a chaperoning activity which is not energy-dependent are referred to as minichaperones.

*Unfolded.* As used herein, a polypeptide may be unfolded when at least part of it has not yet acquired its correct or desired secondary or tertiary structure. A polypeptide is *misfolded* when it has acquired an at least partially incorrect or undesired secondary or tertiary structure.

*Immobilised, immobilising.* Permanently attached, covalently or otherwise. In a preferred aspect of the present invention, the term "immobilise", and grammatical variations thereof, refer to the attachment of molecular chaperones or, preferably, foldase polypeptides to a solid phase support using a method as described in WO99/05163.

*Solid (phase) support.* Reagents used in the invention may be immobilised onto solid phase supports. This means that they are permanently attached to an entity which remains in a different (solid) phase from reagents which are in solution. For example, the solid

phase could be in the form of beads, a "DNA chip", a resin, a matrix, a gel, the material forming the walls of a vessel or the like. Matrices, and in particular gels, such as agarose gels, may conveniently be packed into columns. A particular advantage of solid phase immobilisation is that the reagents may be removed from contact with the polypeptide(s) with facility.

*Foldase.* In general terms, a foldase is an enzyme which participates in the promotion of protein folding through its enzymatic activity to catalyse the rearrangement or isomerisation of bonds in the folding polypeptide. They are thus distinct from a molecular chaperone, which bind to polypeptides in unstable or non-native structural states and promote correct folding without enzymatic catalysis of bond rearrangement. Many classes of foldase are known, and they are common to animals, plants and bacteria. They include peptidyl prolyl isomerases and thiol/disulphide oxidoreductases. The invention comprises the use of all foldases which are capable of promoting protein folding through covalent bond rearrangement.

Moreover, as used herein, the term "a foldase" includes one or more foldases. In general, in the present specification the use of the singular does not preclude the presence of a plurality of the entities referred to, unless the context specifically requires otherwise.

*Thiol/disulphide oxidoreductase.* As the name implies, thiol/disulphide oxidoreductases catalyse the formation of disulphide bonds and can thus dictate the folding rate of disulphide-containing polypeptides. The invention accordingly comprises the use of any polypeptide possessing such an activity. This includes chaperone polypeptides, or fragments thereof, which may possess PDI activity (Wang & Tsou, (1998) FEBS lett. 425:382-384). In Eukaryotes, thiol/disulphide oxidoreductases are generally referred to as PDIs (protein disulphide isomerases). PDI interacts directly with newly synthesised secretory proteins and is required for the folding of nascent polypeptides in the endoplasmic reticulum (ER) of eukaryotic cells. Enzymes found in the ER with PDI activity include mammalian PDI (Edman *et al.*, 1985, Nature 317:267, yeast PDI (Mizunaga *et al.* 1990, J. Biochem. 108:848), mammalian ERp59 (Mazzarella *et al.*, 1990, J. Biochem. 265:1094), mammalian prolyl-4-hydroxylase (Pihlajaniemi *et al.* ,



1987, EMBO J. 6: 643) yeast GSBP (Lamantia *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:4453) and mammalian T3BP (Yamauchi *et al.*, 1987, Biochem. Biophys. Res. Commun. 146:1485), *A. niger* PdiA (Ngiam *et al.*, (1997) Curr. genet. 31:133-138) and yeast EUGI (Tachibana *et al.*, 1992, Mol. Cell Biol. 12, 4601). In prokaryotes, equivalent  
 5 proteins exist, such as the DsbA protein of *E. coli*. Other peptides with similar activity include, for example, p52 from *T. cruzi* (Moutiez *et al.*, (1997) Biochem. J. 322:43-48). These polypeptides, and other functionally equivalent polypeptides, are included with the scope of the present invention, as are derivatives of the polypeptides which share the relevant activity (see below). Preferably, the thiol/disulphide oxidoreductase according to  
 10 the invention is selected from the group consisting of mammalian PDI or *E. coli* DsbA.

*Peptidyl-prolyl isomerase.* Peptidyl-prolyl isomerases are known enzymes widely present in a variety of cells. Examples include cyclophilin (see, for example, Bergsma *et al.* (1991) J. Biol. Chem. 266:23204-23214), parbulen, SurA (Rouviere and Gross, (1996)  
 15 Genes Dev. 10:3170-3182) and FK506 binding proteins FKBP51 and FKBP52. PPI is responsible for the *cis-trans* isomerisation of peptidyl-prolyl bonds in polypeptides, thus promoting correct folding. The invention includes any polypeptide having PPI activity. This includes chaperone polypeptides, or fragments thereof, which may possess PPI activity (Wang & Tsou, (1998) FEBS lett. 425:382-384).

20  
*Molecular Chaperone.* Chaperones, or chaperonins, are polypeptides which promote protein folding by non-enzymatic means, in that they do not catalyse the chemical modification of any structures in folding polypeptides, by promote the correct folding of polypeptides by facilitating correct structural alignment thereof. Molecular chaperones  
 25 are well known in the art, several families thereof being characterised. The invention is applicable to any molecular chaperone molecule, which term includes, for example, the molecular chaperones selected from the following non-exhaustive group:

p90 Calnexin	Salopek <i>et al.</i> , J. Investig Dermatol Symp Proc (1996) 1:195
HSP family	Walsh <i>et al.</i> , Cell Mol. Life Sci. (1997) 53:198
HSP 70 family	Rokutan <i>et al.</i> , J. Med. Invest. (1998) 44:137
DNA K	Rudiger <i>et al.</i> , Nat. Struct. Biol. (1997) 4:342

DNAJ	Cheetham <i>et al.</i> , Cell Stress Chaperones (1998) 3:28
HSP 60 family; GroEL	Richardson <i>et al.</i> , Trends Biochem. (1998) 23:138
ER-associated chaperones	Kim <i>et al.</i> , Endocr Rev (1998) 19:173
HSP 90	Smith, Biol. Chem. (1988) 379:283
Hsc 70	Hohfeld, Biol. Chem. (1988) 379:269
sHsps; SecA; SecB	Beissinger <i>et al.</i> , Biol. Chem. (1988) 379:245
Trigger factor	Wang <i>et al.</i> , FEBS Lett. (1998) 425:382
zebrafish hsp 47, 70 and 90	Krone <i>et al.</i> , Biochem. Cell Biol. (1997) 75:487
HSP 47	Nagata, Matrix Biol. (1998) 16:379
GRP 94	Nicchitta <i>et al.</i> , Curr. Opin. Immunol. (1998) 10:103
Cpn 10	Cavanagh, Rev. Reprod. (1996) 1:28
BiP	Sommer <i>et al.</i> , FASEB J. (1997) 11:1227
GRP 78	Brostrom <i>et al.</i> , Prog. Nucl. Acid. res. Mol. Biol. (1998) 58:79
C1p, FtsH	Suzuki <i>et al.</i> , Trends Biochem. Sci. (1997) 22:118
Ig invariant chain	Weenink <i>et al.</i> Immunol. Cell biol. (1997) 75:69
mitochondrial hsp 70	Horst <i>et al.</i> , BBA (1997) 1318:71
EBP	Hinek, Arch. Immunol. Ther. Exp. (1997) 45:15
mitochondrial m-AAA	Langer <i>et al.</i> , Experientia (1996) 52:1069
Yeast Ydj1	Lyman <i>et al.</i> , Experientia (1996) 52:1042
Hsp 104	Tuite <i>et al.</i> , Trends Genet. (1996) 12:467
ApoE	Blain <i>et al.</i> , Presse Med. (1996) 25:763
Syc	Wattiau <i>et al.</i> , Mol. Microbiol. (1996) 20:255
Hip	Ziegelhoffer <i>et al.</i> , Curr. Biol. (1996) 6:272
TriC family	Hendrick <i>et al.</i> , FASEB J. (1995) 9:1559
CCT	Kubota <i>et al.</i> , Eur. J. Biochem. (1995) 230:3
PapD, calmodulin	Stanfield <i>et al.</i> , Curr. Opin. Struct. Biol. (1995) 5:103

Two major families of protein folding chaperones which have been identified, the heat shock protein 60 (hsp60) class and the heat shock protein 70 (hsp70) class, are especially preferred for use herein. Chaperones of the hsp-60 class are structurally distinct from

chaperones of the hsp-70 class. In particular, hsp-60 chaperones appear to form a stable scaffold of two heptamer rings stacked one atop another which interacts with partially folded elements of secondary structure. On the other hand, hsp-70 chaperones are monomers of dimers and appear to interact with short extended regions of a polypeptide.

5

Hsp70 chaperones are well conserved in sequence and function. Analogues of hsp-70 include the eukaryotic hsp70 homologue originally identified as the IgG heavy chain binding protein (BiP). BiP is located in all eukaryotic cells within the lumen of the endoplasmic reticulum (ER). The prokaryotic DnaK hsp70 protein chaperone in  
10 *Escherichia coli* shares about 50% sequence homology with an hsp70 KAR2 chaperone in yeast (Rose *et al.* 1989 Cell 57:1211-1221). Moreover, the presence of mouse BiP in yeast can functionally replace a lost yeast KAR2 gene (Normington *et al.* 19: 1223-1236).

Hsp-60 chaperones are universally conserved (Zeilstra-Ryalls *et al.*, (1991) Ann. Rev.  
15 Microbiol. 45:301-325) and include hsp-60 homologues from large number of species, including man. They include, for example, the *E. coli* GroEL polypeptide; *Ehrlichia sennetsu* GroEL (Zhang *et al.*, (1997) FEMS Immunol. Med. Microbiol. 18:39-46); *Trichomonas vaginalis* hsp-60 (Bozner *et al.*, (1997) J. Parasitol. 83:224-229; rat hsp-60 (Venner *et al.*, (1990) NAR 18:5309; and yeast hsp-60 (Johnson *et al.*, (1989) Gene  
20 84:295-302.

In a preferred aspect, the present invention relates to fragments of polypeptides of the hsp-60 family. These proteins being universally conserved, any member of the family may be used; however, in a particularly advantageous embodiment, fragments of GroEL,  
25 such as *E. coli* GroEL, are employed. It has also found that agarose-immobilised calmodulin does have a chaperoning activity, presumably because of its exposed hydrophobic groups.

The sequence of GroEL is available in the art and from academic databases (see GenBank  
30 Accession No. P06159); however, GroEL fragments which conform to the database sequence are inoperative. Specifically, the database contains a sequence in which positions 262 and 267 are occupied by Alanine and Isoleucine respectively. Fragments

incorporating one or both of these residues at these positions are inoperative and unable to promote the folding of polypeptides. The invention, instead, relates to a GroEL polypeptide in which at least one of positions 262 and 267 is occupied by Leucine and Methionine respectively.

5

*Derivative.* The present invention relates to derivatives of molecular chaperones, peptidyl-prolyl isomerases and thiol/disulphide oxidoreductases. In a preferred aspect, therefore, the terms "molecular chaperone", "peptidyl-prolyl isomerase" and "thiol-disulphide oxidoreductase" include derivatives thereof which retain the stated activity.

10 The derivatives provided by the present invention include splice variants encoded by mRNA generated by alternative splicing of a primary transcript, amino acid mutants, glycosylation variants and other covalent derivatives of molecular chaperones or foldases which retain the functional properties of molecular chaperones, peptidyl-prolyl isomerases and/or thiol/disulphide oxidoreductases. Exemplary derivatives include molecules which

15 are covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Such a moiety may be a detectable moiety such as an enzyme or a radioisotope. Further included are naturally occurring variants of molecular chaperones or foldases found within a particular species, whether mammalian, other vertebrate, yeast, prokaryotic or otherwise. Such a variant may

20 be encoded by a related gene of the same gene family, by an allelic variant of a particular gene, or represent an alternative splicing variant of a molecular chaperone or foldase. Possible derivatives of the polypeptides employed in the invention are described below.

### Description of Preferred Embodiments

In a first aspect, the present invention relates to a method for oligomerising polypeptides and novel oligomeric polypeptides producible thereby. By incorporating polypeptides  
5 into a scaffold which is capable of oligomerising, as described herein, oligomers may be produced wherein the chosen polypeptides are juxtaposed. The polypeptides selected for oligomerisation may be the same or different; thus, it is possible to produce homooligomers or heterooligomers.

10 Preferably, the oligomeric proteins of the invention are oligomeric minichaperone polypeptides. Oligomeric minichaperones have been shown to possess particularly advantageous properties.

The invention thus relates to the use of oligomeric molecular chaperone fragments,  
15 whether alone or in combination with other polypeptides, for promoting the folding or refolding of polypeptides.

The present invention may be practised in a number of configurations, according to the required use to which the invention is to be put. In a first configuration, the invention  
20 relates to the use of minichaperone oligomers alone to promote the folding or refolding of polypeptides. This may be performed *in vivo* or *in vitro*, in solution or on a solid support. For example, oligomerised minichaperones may be immobilised onto resins and packed into columns for use in refolding polypeptides which are passed through the column. Methods for immobilising minichaperones are described in International patent  
25 application WO99/05163, incorporated herein by reference.

In an alternative configuration, minichaperone oligomers according to the invention may be expressed *in vivo* or administered to cells or organisms *in vivo* in order to promote protein folding therein.  
30

In a second configuration, the invention provides a combination of a molecular chaperone and a thiol/disulphide oxidoreductase to facilitate protein folding. The combination of a

molecular chaperone and a thiol/disulphide oxidoreductase provides a synergistic effect on protein folding which results in a greater quantity of active, correctly folded protein being produced than would be expected from a merely additive relationship. Advantageously, one or more of the components used to promote protein folding in accordance with the present invention is immobilised on a solid support. However, both molecular chaperones and thiol/disulphide oxidoreductases may be used in solution. They may be used in free solution, but also in suspension, for example bound to a matrix such as beads, for example Sepharose beads, or bound to solid surfaces which are in contact with solutions, such as the inside surfaces of bottles containing solutions, test tubes and the like.

In a third configuration, the invention relates to the use of a combination of a molecular chaperone and a thiol/disulphide oxidoreductase with a peptidyl prolyl isomerase. The peptidyl prolyl isomerase may be present either bound to a solid support, or in solution. Moreover, it may be bound to beads suspended in solution. The peptidyl prolyl isomerases may be used together with a molecular chaperone alone, with a thiol/disulphide oxidoreductase alone, or with both a molecular chaperone and a thiol/disulphide oxidoreductase. In the latter case, further synergistic effects are apparent over the additive effects which would be expected from the use of the three components together. In particular, an increase in the proportion of the folded protein which is recovered as monodisperse protein, as opposed to aggregated protein, increases substantially.

Used in accordance with any of the foregoing configurations, or otherwise in accordance with the following claims, the invention may be used to facilitate protein folding in a variety of situations. For example, the invention may be used to assist in refolding recombinantly produced polypeptides, which are obtained in an unfolded or misfolded form. Thus, recombinantly produced polypeptides may be passed down a column on which is immobilised a composition comprising protein disulphide isomerase and/or a molecular chaperone and/or a prolyl peptidyl isomerase.

In an alternative embodiment, the invention may be employed to maintain the folded conformation of proteins, for example during storage, in order to increase shelf life.

under storage conditions, many proteins lose their activity, as a result of disruption of correct folding. The presence of molecular chaperones, in combination with foldases, reduces or reverses the tendency of polypeptides to become unfolded and thus greatly increases the shelf life thereof. In this embodiment, the invention may be applied to  
5 reagents which comprise polypeptide components, such as enzymes, tissue culture components, and other proteinaceous reagents stored in solution.

In a further embodiment, the invention may be used to promote the correct folding of proteins which, through storage, exposure to denaturing conditions or otherwise, have  
10 become misfolded. Thus, the invention may be used to recondition reagents or other proteins. For example, proteins in need of reconditioning may be passed down a column to which is immobilised a combination of reagents in accordance with the invention. Alternatively, beads having immobilised thereon such a combination may be suspended in a solution comprising the proteins in need of reconditioning. Moreover, the components  
15 of the combination according to the invention may be added in solution to the proteins in need of reconditioning.

As noted above, the components of the combination according to the invention may comprise derivatives of molecular chaperones or foldases, including variants of such  
20 polypeptides which retain common structural features thereof. Variants which retain common structural features can be fragments of molecular chaperones or foldases. Fragments of molecular chaperones or foldases comprise smaller polypeptides derived from therefrom. Preferably, smaller polypeptides derived from the molecular chaperones or foldases according to the invention define a single feature which is characteristic of the  
25 molecular chaperones or foldases. Fragments may in theory be almost any size, as long as they retain the activity of the molecular chaperones or foldases described herein.

With respect to molecular chaperones of the GroEL/hsp-60 family, a preferred set of fragments have been identified which possess the desired activity. These fragments are  
30 set forth in our copending international patent application PCT/GB96/02980 and in essence comprise any fragment comprising at least amino acid residues 230-271 of intact GroEL, or their equivalent in another hsp-60 chaperone. Preferably, the fragments should

not extend beyond residues 150-455 or 151-456 of GroEL or their equivalent in another hsp-60 chaperone. Where the fragments are GroEL fragments, they must not possess the mutant GroEL sequence as set forth above; in other words, they must not have an Alanine residue at position 262 and/or an Isoleucine residue at position 267 of the sequence of  
5 intact GroEL.

Advantageously, the fragments comprise the apical domain of GroEL, or its equivalent in other molecular chaperones, or a region homologous thereto as defined herein. The apical domain spans amino acids 191-376 of intact GroEL. This domain is found to be  
10 homologous amongst a wide number of species and chaperone types.

Preferably, molecular chaperones according to the invention are homologous to, or are capable of hybridising under stringent conditions with, a region corresponding to the apical domain of GroEL as defined above.  
15

In a highly preferred embodiment, the fragments are selected from the group consisting of residues 191-376, 191-345 and 191-335 of the sequence of intact GroEL.

Derivatives of the molecular chaperones or foldases also comprise mutants thereof, including mutants of fragments and other derivatives, which may contain amino acid deletions, additions or substitutions, subject to the requirement to maintain the activity of the molecular chaperones or foldases described herein. Thus, conservative amino acid substitutions may be made substantially without altering the nature of the molecular chaperones or foldases, as may truncations from the 5' or 3' ends. Deletions and  
20 substitutions may moreover be made to the fragments of the molecular chaperones or foldases comprised by the invention. Mutants may be produced from a DNA encoding a molecular chaperone or foldase which has been subjected to in vitro mutagenesis resulting e.g. in an addition, exchange and/or deletion of one or more amino acids. For example, substitutional, deletional or insertional variants of molecular chaperones or foldases can  
25 be prepared by recombinant methods and screened for immuno-crossreactivity with the native forms of the relevant molecular chaperone or foldase.  
30



The fragments, mutants and other derivative of the molecular chaperones or foldases preferably retain substantial homology with the native molecular chaperones or foldases. As used herein, "homology" means that the two entities share sufficient characteristics for the skilled person to determine that they are similar in origin and function. Preferably, 5 homology is used to refer to sequence identity. Thus, the derivatives of molecular chaperones or foldases preferably retain substantial sequence identity with native forms of the relevant molecular chaperone or foldase.

10 In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least 20, preferably 30 amino of the minichaperone. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for the chaperone activity rather than non-essential neighbouring sequences. Although homology can also be considered in 15 terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

20 Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

25 % homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

30 Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most

sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

5

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see <http://www.ncbi.nih.gov/BLAST/>), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410; FASTA is available for online searching at, for example, <http://www2.ebi.ac.uk.fasta3>) and the GENEWORKS suite of comparison tools. However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of

programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

5

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

- 10 Alternatively, sequence similarity may be defined according to the ability to hybridise to a complementary strand of a chaperone or foldase sequence as set forth above.

Preferably, the sequences are able to hybridise with high stringency. Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such  
 15 conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature ( $T_m$ ) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is performed under conditions of higher  
 20 stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M Na<sup>+</sup> at 65-68 °C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution  
 25 containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 Na<sup>+</sup> pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

- 30 Moderate stringency refers to conditions equivalent to hybridisation in the above described solution but at about 60-62°C. In that case the final wash is performed at the hybridisation temperature in 1x SSC, 0.1 % SDS.

Low stringency refers to conditions equivalent to hybridisation in the above described solution at about 50-52°C. In that case, the final wash is performed at the hybridisation temperature in 2x SSC, 0.1 % SDS.

5

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, et al., eds. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York or Ausubel, et al., eds. (1990) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the probe also play a role.

15 The invention also envisages the administration of polypeptide oligomers according to the invention as compositions, preferably for the treatment of diseases associated with protein misfolding. The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (e.g. using slow release molecules). Depending on the route of administration, the active ingredient may be required to be  
20 coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredient.

In order to administer the combination by other than parenteral administration, it will be coated by, or administered with, a material to prevent its inactivation. For example, the  
25 combination may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin.

30

Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes.

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations  
5 contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be  
10 sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like),  
15 suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various  
20 antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

25 Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the sterilised active ingredient into a sterile vehicle which  
30 contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying

technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the combination of polypeptides is suitably protected as described above, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for

pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

5

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to  
10 produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such as active material for the treatment of disease in living subjects having  
15 a diseased condition in which bodily health is impaired.

The principal active ingredients are compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form. In the case of compositions containing supplementary active  
20 ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

In a further aspect there is provided the combination of the invention as hereinbefore defined for use in the treatment of disease. Consequently there is provided the use of a  
25 combination of the invention for the manufacture of a medicament for the treatment of disease associated with aberrant protein/polypeptide structure. The aberrant nature of the protein/polypeptide may be due to misfolding or unfolding which in turn may be due to an anomalous e.g. mutated amino acid sequence. The protein/polypeptide may be destabilised or deposited as plaques e.g. as in Alzheimer's disease. The disease might be  
30 caused by a prion. A polypeptide-based medicament of the invention would act to renature or resolubilise aberrant, defective or deposited proteins.

The invention is further described below, for the purposes of illustration only, in the following examples.

## Examples

5

### 1. General Experimental Procedures

**Bacterial and bacteriophage strains.** The *E. coli* strains used in this study were: C41(DE3), a mutant of BL21(DE3) capable of expressing toxic genes (29); SV2 (B178*groEL44*), SV3 (B178*groEL59*) and SV6 (B178*groEL673*): isogenic strains carrying temperature-sensitive alleles of *groEL*; SV1(=B178) (30), AI90 ( $\Delta$ *groEL::kan<sup>R</sup>*) [pBAD-EL] (15), and TG1 (31). Bacteriophage  $\lambda$  b2cI (14) was used according to standard methods (32); plaque formation was assayed at 30 °C. T4D0, a derivative of bacteriophage T4 (14), was used according to standard methods (33); plaque formation was assayed at 37 °C.

**Plasmid constructions.** Standard molecular biology procedures were used (34). The schematic organisation of the plasmids used in this study is represented Figure 2. *Gp31* gene was PCR amplified using two oligonucleotides 5' – C TTC AGA CAT ATG TCT GAA GTA CAA CAG CTA CC – 3' and 5' – TAA CGG CCG TTA CTT ATA AAG ACA CGG AAT AGC – 3' producing a 358 bp DNA using pSV25 (26) as template. The DNA sequence of the mobile loop of Gp31 (residues 23 to 44) was removed by PCR, as described (35), using oligonucleotides 5' – GGA GAA GTT CCT GAA CTG – 3' and 5' – GGA TCC GGC TTG TGC AGG TTC – 3', creating a unique *BamH* I site (bold characters).

*GroEL* gene minichaperone (corresponding to the apical domain of GroEL, residues 191 to 376; (13)) was amplified by PCR using oligonucleotides, containing a *BamH* I site (underlined), 5' – TTC GGA TCC GAA GGT ATG CAG TTC GAC C – 3' and 5' – GTT GGA TCC AAC GCC GCC TGC CAG TTT C – 3' and cloned into the unique *BamH* I site of pRSETA-Gp31 $\Delta$ loop vector, inserting minichaperone GroEL(191-376) in frame into Gp31 $\Delta$ loop sequence.



*GroEL*(E191G; *groEL44* allele) gene was PCR amplified from *E. coli* SV2 strain (14) using two oligonucleotides 5' – T AGC TGC CAT ATG GCA GCT AAA GAC GTA AAA TTC GG – 3' and 5' – ATG TAA CGG CCG TTA CAT CAT GCC GCC CAT  
 5 GCC ACC – 3' producing a 1,659 bp DNA with unique sites for *Nde* I and *Eag* I (underlined).

The different genes were subcloned into the unique *Nde* I and *Eag* I unique sites of pACYC184, pJC and pBAD30 (36) vectors (16). A colony-based PCR procedure was  
 10 used to identify the positive clones (37). PCR cycle sequencing using fluorescent dideoxy chain terminators (Applied Biosystems) were performed and analysed on an Applied Biosystems 373A Automated DNA. All PCR amplified DNA fragments were sequenced after cloning.

15 **Protein expression, purification and characterisation.** The GroE proteins, ~57.5 kDa GroEL and ~10 kDa GroES, were expressed and purified as previously described (16, 38). GroEL(E191G) protein was expressed by inducing the *P<sub>BAD</sub>* promoter of pBAD30 based vector with 0.2 % arabinose in *E. coli* SV2 strain (14). Purification was performed essentially as described (38). The over-expression of histidine-tagged (short histidine tail;  
 20 sht)-minichaperone GroEL(191-376) in *E. coli* C41(DE3) cells and, the purification and the removal of sht by thrombin cleavage were carried out essentially as previously described (13).

Gp31 proteins wild-type (~12 kDa),  $\Delta$ loop (~10.4 kDa) and MC<sub>7</sub> (~30.6 kDa), were  
 25 expressed by inducing the T7 promoter of pRSETA-*Eag* I based vectors with isopropyl- $\beta$ -D-thiogalactoside (IPTG) in *E. coli* C41(DE3) (29) overnight at 25 °C. Purification procedures were essentially as described (26, 39). Ammonium sulphate precipitation (only 20% saturation for  $\Delta$ loop; 30 to 70% saturation for wild-type and MC<sub>7</sub>) was followed by ion-exchange chromatography on a DEAE-Sepharose column (Pharmacia  
 30 Biotech.). Gp31 proteins were eluted with a 0-0.5 M NaCl gradient in 20 mM Tris-HCl, 1 mM EDTA, 1 mM  $\beta$ -mercaptoethanol, pH 7.5;  $\Delta$ loop and MC<sub>7</sub> eluted between 0.32-0.44 and 0.38-0.48 mM NaCl, respectively. Gp31 proteins were further purified by gel

filtration chromatography on a Superdex™ 200 (HiLoad 26/10) column (Pharmacia Biotech.) equilibrated with 100 mM Tris-HCl, pH 7.5 and, dialysed against and stored in 50 mM Tris-HCl, 0.1 mM EDTA, 1 mM  $\beta$ -mercaptoethanol, pH 7.5.

- 5 Proteins were analysed by electrospray mass spectrometry. Protein concentration was determined by absorbance at 276 nm using the method of Gill & von Hippel (40) and confirmed by quantitative amino acid analysis.

10 Constitutive expression under the control of the tetracycline-resistance gene promoter / operator was obtained using the high copy-number pJC vectors (16). pBAD30 vector allows inducible expression with 0.2-0.5 % arabinose controlled by the  $P_{BAD}$  promoter and its regulatory gene, *araC* (36). The level of expression of MC<sub>7</sub> was analysed by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions followed by Western blotting as described (16).

15

**Molecular weight determination by analytical gel filtration chromatography.** 100  $\mu$ l aliquots of protein (1 mg ml<sup>-1</sup>) were loaded onto a Superdex™ 200 (HR 10/30) column (Pharmacia Biotech.) equilibrated with 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 at 0.5 ml min<sup>-1</sup>. The column was calibrated using gel filtration standards from Pharmacia Biotech.  
 20 (thyroglobulin, MW=669 kDa; ferritin, MW=440 kDa; aldolase, MW=158 kDa; ovalbumin, MW=45 kDa; chymotrypsinogen MW=25 kDa; RNase, MW=13 kDa). Molecular weights were determined by logarithmic interpolation.

**Circular dichroism spectroscopy (CD).** Far UV (200-250 nm)-CD spectra at 25 °C  
 25 were measured on a Jasco J720 spectropolarimeter interfaced with a Neslab PTC-348WI water bath, using a thermostatted cuvette of 0.1 cm path length. Spectra are averages of 10 scans and were recorded with a sampling interval of 0.1 nm. Thermal denaturation was carried out from 5-95 °C at a linear rate of 1 °C min<sup>-1</sup> and monitored at 222 nm. The reversibility was checked after incubation at 95 °C for 20 min and cooling to and  
 30 equilibration at 5 °C. The protein concentration was 45  $\mu$ M in 10 mM sodium phosphate buffer pH 7.8, 2.5 mM DTE (dithioerythritol).

GroES binding and competition assays by ELISA. Proteins were coated onto plastic microtitre plates (Maxisorb, Nunc) overnight at 4 °C at a concentration of 10 mg/ml in carbonate buffer (50 mM NaHCO<sub>3</sub>, pH 9.6). Plates were blocked for 1 hour at 25 °C with 2% Marvel in PBS (phosphate buffered saline: 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, pH 7.0).

5 GroES, at 10 mg/ml in 100 ml of 10 mM Tris-HCl, 200 mM KCl, pH 7.4, were bound at 25 °C for 1 hour. Bound GroES were detected with rabbit anti-GroES antibodies (Sigma) followed by anti-rabbit immunoglobulins horseradish peroxidase conjugated antibodies (Sigma).

10 A peptide corresponding to the mobile loop of GroES (residues 16 to 32, numbered as in (2)) was synthesised by conventional chemistry. The inhibition of the binding of MC<sub>7</sub> proteins by the free peptide was analysed by ELISA, essentially as above, by adding different concentrations (between 10,000 to 0.1 mM) of free peptide solved in 0.1% TFA solution to 1 mg of proteins prior incubation to coated GroES proteins (10 mg/ml).

15 GroEL molecules were detected with rabbit anti-GroEL antibodies (Sigma) followed by anti-rabbit immunoglobulins horseradish peroxidase conjugate antibodies (Sigma). ELISAs were developed with 3',3',5',5'-tetramethylbenzidine (TMB, Boehringer Mannheim). Reactions were stopped with 50 ml of 1M H<sub>2</sub>SO<sub>4</sub> after 10 min and readings taken by subtracting the *A*<sub>650 nm</sub> from the *A*<sub>450 nm</sub>.

20

*In vivo* complementation experiments. Complementation experiments were performed by transforming electro-competent SV2 or SV6 cells with the pJC series of expression vectors and plating an aliquot of the transformation reactions directly at 43 °C. The percentage of viable cells relative to the growth at 30 °C was determined.

25 representative number of clones which grew at 43 °C were incubated in absence of any selective markers at permissive temperature. After prolonged growth the loss of the pJC plasmids and the ts phenotype were verified.

P1 transduction (41), using strain AI90 ( $\Delta groEL::kan^R$ ) [pBAD-EL] as donor (15), was used to delete the *groEL* gene of TG1 cells transfected by the different pJC vectors. Transductants were selected on LB plates containing 10  $\mu\text{g/mL}$  of kanamycin at 37 °C. Approximately 25 colonies were transferred onto plates containing kanamycin at 50  $\mu\text{g/mL}$ . After incubation for 24 h at 37 °C, colonies that grew were screened by PCR as described (15).

AI90 ( $\Delta groEL::kan^R$ ) [pBAD-EL] cells were transformed with the pJC vector series. Transformants were selected at 37 °C on LB supplemented with 50  $\mu\text{g mL}^{-1}$  of kanamycin, 120  $\mu\text{g mL}^{-1}$  of ampicillin, 25  $\mu\text{g mL}^{-1}$  of chloramphenicol and 0.2% L(+)-arabinose. Depletion of GroEL protein was analysed at 37 °C by plating the same quantity of AI90 [pBAD-EL + pJC vectors] cells on LB plates containing 1% D(+)-glucose or various amount of arabinose.

Each experiment was performed in triplicate. Plasmids carrying no *groE* genes or encoding the GroE proteins were used as negative or positive controls, respectively.

**Effect on Lorist6 replication of over-expressing of MC<sub>7</sub>.** The effect of over-expressing Gp31 proteins from pJC vector series on the replication of the bacteriophage  $\lambda$  origin vector, Lorist6 (42) in TG1 (31) cells was determined essentially as described (16).

## 2. Example 1: Gp31 protein as a scaffold for displaying heptameric GroEL minichaperone.

We describe a scaffold on which any polypeptide may be hung; as a result, the polypeptide is oligomerised. The scaffold is the bacteriophage T4 Gp31 (gene product) heptamer. The monomeric protein is 12 kDa, but it spontaneously forms a stable heptameric structure (90 kDa) of which the three-dimensional structure is known from X-ray crystallography (27). This illustrates that a highly mobile polypeptide loop (residues 27 to 42; (28)) projects from each subunit (Figure 1). The basis of the method is the substitution of this loop by a chosen peptide sequence.

In an effort to increase the avidity of minichaperones for substrates, and consequently to improve their chaperonin-facilitated protein folding, we generated the fusion protein, Gp31 $\Delta$ loop::GroEL(191-376) (hereafter named MC<sub>7</sub>), where the mobile loop of Gp31 was replaced by the sequence of minichaperone GroEL (residues 191 to 376) (Figure 2).

MC<sub>7</sub> was cloned downstream of the T7 promoter of pRSETasht-*Eag* I vector (16). After sonication, the soluble and insoluble fractions of IPTG-induced transfected C41(DE3) cells (29) were analysed by SDS-PAGE. Most of MC<sub>7</sub> was present in the insoluble fraction. Insoluble material dissolved in 8 M urea was efficiently refolded by dialysis at 4 °C. MC<sub>7</sub> was purified by ion-exchange and gel filtration chromatography. MC<sub>7</sub> was over-expressed in C41(DE3) cells to give 0.25-0.5 g purified protein per L of culture. Purified MC<sub>7</sub> coincided to seven 30.6 kDa subunits of Gp31 $\Delta$ loop::GroEL(191-376) as determined by analytical gel filtration chromatography (Figure 3); Gp31 $\Delta$ loop corresponds to a tetra-decamer (14 subunits). The preliminary electronic microscopy studies of MC<sub>7</sub> revealed views that could correspond to front views of oligomers with a diameter identical to the one of GroEL (J.L. Carrascosa, J.C. & A.R.F., unpublished). The circular dichroism spectrum of MC<sub>7</sub> indicated significant  $\alpha$ -helical structure (Figure 4a). The thermal unfolding monitored by far UV-CD was reversible although more than one transition exist (Figure 4b).

### 3. Example 2: Binding to heptameric bacterial co-chaperonin, GroES.

The functionality of MC<sub>7</sub> was examined for binding to GroES, since the interaction between GroEL and GroES is known to be less favourable for one monomer than for the heptamer. MC<sub>7</sub> bound specifically to GroES, conversely monomeric minichaperone GroEL(191-376) did not detectably bind the bacterial co-chaperonin (Figure 5a). The avidity effect of the heptameric structure of MC<sub>7</sub> was confirmed by analysing the binding of antibodies specific to GroEL (data not shown).

The ability of a synthetic peptide corresponding to residues 16 to 22 of GroES mobile loop to displace bound GroES from MC<sub>7</sub> was tested by competition ELISA. The synthetic GroES mobile loop peptide (residues 16 to 22) did inhibit the binding of MC<sub>7</sub>

with an  $IC_{50}$  of 10  $\mu$ M compared to 100  $\mu$ M for GroEL (Figure 5b). The apparent dissociation constant for the formation of the GroEL-GroES complex is low ( $10^{-6}$  M), which is compatible with cycling of GroES on and off GroEL during chaperonin-assisted folding (43). On the other hand, GroEL<sub>SR1</sub> (44) is unable to release GroES in the absence of signal transmitted via the binding of ATP to an adjacent ring (8, 9, 44). The 10-fold decrease of the affinity of MC<sub>7</sub> for GroES may be sufficient for multiple binding and release cycles.

#### 4. Example 3: *In vivo* complementation of thermosensitive *groEL* mutant alleles at 43 °C.

We sought complementation of two thermosensitive (*ts*) *groEL* mutants of *E. coli* at 43 °C. *E. coli* SV2 has the mutation Glu191→Gly in GroEL corresponding to *groEL44* allele (14), while SV6 carries the *EL673* allele, which has two mutations, Gly173→Asp and Gly337→Asp (14). Complementation experiments were performed by transforming the thermosensitive (*ts*) *E. coli* strains SV2 or SV6 with the pJC series of expression vectors (see Figure 2; (16)) and plating an aliquot of the transformation reaction directly at 43 °C. Subsequently, plasmids pJC from a representative number of individual clones growing at 43 °C were lost in the absence of continued chloramphenicol selection. Nearly all (= 95%) the cured clones were thermosensitive at 43 °C indicating the absence of recombination events for the reconstitution of wild-type *groEL* gene.

The results obtained are qualitatively similar to those previously described (16). The defective *groEL* in SV6 was complemented by expression of minichaperone sht-GroEL(191-345), and less well by sht-GroEL(193-335). The converse is true for SV2. MC<sub>7</sub> complements both temperature-sensitive *E. coli* *groEL44* and *groEL673* alleles at 43 °C (Table 1) better than the minichaperones. Colony forming units were not observed for either strain at 43 °C with vectors either lacking inserts (pJC<sub>sht</sub>) or containing MC<sub>7</sub> lacking the GroEL(191-376) inserts (pJCGp31 $\Delta$ loop).

It was speculated the higher stability of shortest minichaperone sht-GroEL(193-335) could be responsible for the complementation of *groEL44* mutant allele (16). To test this

eventuality, we purified GroEL(E191G; *groEL44* allele) mutant and compared its thermal stability with the wild-type GroEL. We found no difference in stability between the mutant and the wild-type proteins (data not shown). In addition, highly stable functional mutants of GroEL (193-345) (Q. Wang & A.R.F., unpublished) do not complement as well as do the parental minichaperone (Table 1), the defects in SV2 or even SV6 (data not shown). We concluded the thermal stability of minichaperone does not account for the complementation of *groEL* defects.

Table 1. Relative colony forming ability of transformed *ts groEL44* or *groEL673* *E. coli* strains at 43 °C.

		Plasmids pJC									
<i>groE</i> strains	short his tag (sht) ( <i>ES</i> <sup>+</sup> , <i>EL</i> <sup>-</sup> )	GroES (1-97)	Gp31 (1-111)	Gp31 ?loop	GroEL (1-548)	GroESL	sht-GroEL (191-376)	sht-GroEL (191-345)	sht-GroEL (193-345)	sht-GroEL (193-335)	Gp31?:: GroEL (191-376)
SV2 <i>groEL44</i>	< 10 <sup>-4</sup>	5 x 10 <sup>-3</sup>	0.5 x 10 <sup>-3</sup>	< 10 <sup>-4</sup>	1	1	< 10 <sup>-4</sup>	0.01-0.02	1-2 x 10 <sup>-4</sup>	0.05-0.09	0.15-0.2
SV6 <i>groEL673</i>	< 10 <sup>-4</sup>	< 10 <sup>-4</sup>	< 10 <sup>-4</sup>	< 10 <sup>-4</sup>	1	1	< 10 <sup>-4</sup>	0.07-0.09	< 10 <sup>-4</sup>	0.03-0.05	0.1



#### 5. Example 4: *In vivo* complementation at 37 °C.

5 The effects of MC<sub>7</sub> on the growth at 37 °C of a strain of *E. coli* in which the chromosomal *groEL* gene had been deleted were analysed in two ways. First, we attempted to delete the *groEL* gene of TG1 which had been transformed with the different pJC MC<sub>7</sub> vector by P1 transduction (15, 16). However, no transductants could be obtained where the *groEL* gene had been deleted, unless intact GroEL was expressed from the complementing  
 10 plasmid. This is consistent with the known essential role of GroEL (1). Second, we analysed the complementation of AI90 ( $\Delta groEL::kan^R$ ) [pBAD-EL] *E. coli* strain (15). In this strain, the chromosomal *groEL* gene has been deleted and GroEL is expressed exclusively from a plasmid-borne copy of the gene which can be tightly regulated by the arabinose *P<sub>BAD</sub>* promoter and its regulatory gene, *araC* (36). AraC protein acts as either a  
 15 repressor or an activator depending on the carbon source used. *P<sub>BAD</sub>* is activated by arabinose but repressed by glucose (36). The AI90 [pBAD-EL] cells can not grow on medium supplemented with glucose at 37 °C (15). As with minichaperones (16), MC<sub>7</sub> was unable to suppress this *groEL* growth defect (Table 2). We then determined whether MC<sub>7</sub> could supplement low levels of GroEL from transfected AI90 [pBAD-EL]. At 0.01%  
 20 arabinose, cells transfected with pJC expressing *sht* alone, Gp31 $\Delta$ loop or *sht*-GroEL(191-376), showed little colony forming ability (less than 5%). But those containing pJC MC<sub>7</sub> produced about 30% of the number produced in the presence of 0.2% arabinose. Thus, pJC MC<sub>7</sub> can significantly supplement depleted levels of GroEL, about twice as effectively as pJC *sht*-GroEL(193-335) (16).

**Table 2.** Plating ability of transformed AI90 (*groEL::kan*) [pBADEL] *E. coli* strain at 37 °C in presence of different amount of arabinose.

<i>Plasmids pJC</i>	% L(+)arabinose			
	0.15	0.10	0.01	0.00
short his tag (sht) ( <i>ES</i> <sup>-</sup> , <i>EL</i> <sup>-</sup> )	++	+	+/-	-
Gp31?loop	++	+	+/-	-
GroEL (1-548)	+++	+++	+++	+++
sht-GroEL (191-376)	++	+	+/-	-
Gp31?::	+++	+++	+	-
GroEL (191-376)				

+++, growth identical to that in presence of 0.2 % L(+)arabinose (100 %), in terms of both  
 5 number and size; ++, about 50 % of the colonies relative to that in presence of 0.2 %  
 L(+)arabinose; +, about 30 % of the colonies; +/-, = 5 % of the colonies and size reduced  
 relative to that in presence of 0.2 % L(+)arabinose; -, no visible colonies.

## 10 6. Example 5: Effect on bacteriophages $\lambda$ and T4 growth of over-expressing MC<sub>7</sub>.

Bacteriophages  $\lambda$  and T4 require the chaperonins GroES and GroEL for protein folding  
 during morphogenesis (45). Nine *groE* alleles which fail to support  $\lambda$  growth have been  
 15 sequenced (14). We examined the ability of MC<sub>7</sub>, over-expressed from the constitutive  
 tet promoter on a high-copy number vector (see Figure 2; (16)), to complement three  
 mutant *groEL* alleles for plaque formation by  $\lambda$  (b2cI) at 30 °C (Table 3) and T4 at 37 °C

(Table 4). The *groE* operon was named for its effects on the E protein of  $\lambda$  (30). Although heat induction of the *groE* operon has been shown to decrease burst size of  $\lambda$  bacteriophage in *E. coli* (46). In contrast, we showed that the over-expression of GroEL alone, which resulted in slower growth of the bacteria, suffices to inhibit  $\lambda$  growth (Table 3). This effect was specific; over-expression of GroEL together with GroES caused only a four-fold drop in plaques. Over-expression of GroES alone had no effect. Minichaperone GroEL(191-376) had no effect on plaque counts in SV1 (*groE*<sup>+</sup>). Conversely, over-expression of MC<sub>7</sub> prevents plaque formation by bacteriophage  $\lambda$  in SV1, but less markedly than GroEL (Table 3). It seems that the main effect of GroEL over-expression is mediated through the  $\lambda$  origin, which requires two proteins, O and P (16, 46). As GroEL (16), MC<sub>7</sub> (or GroEL<sub>SR1</sub>) inhibit the replication of the Lorist6 plasmid which use the bacteriophage  $\lambda$  origin (data not shown). The effect on Lorist6 shows that the unfoldase activity is also an essential part of GroEL activity *in vivo*. MC<sub>7</sub> and minichaperones (16) possess both, unfolding and folding, activities.

GroEL over-expression gives weak complementation of  $\lambda$  growth in SV2 (*groEL44*) and SV3 (*groEL59*; Ser201→Phe). MC<sub>7</sub> does not complement any of the *E. coli groEL* mutant strains for bacteriophage  $\lambda$  growth at 30 °C (Table 3).

Bacteriophage T4 (T4D0) also requires a functional *groEL* gene, but encodes a protein Gp31 which can substitute for GroES (25, 26). The requirement for GroEL can be distinguished genetically from  $\lambda$ 's requirement. Thus only two of the four *groEL* alleles fail to support T4 replication; these are also the two thermosensitive mutations *EL44* and *EL673* (14, 45). While over-expression of Gp31 allows T4 growth in all strains (only SV2 and SV6 strains normally do not allow T4 growth), over-expression of Gp31 $\Delta$ loop inhibits T4 replication. On the other hand, MC<sub>7</sub> does complement *E. coli groEL* mutant strains for bacteriophage T4 growth at 30°C (Table 3).

Surprisingly, over-expression of GroES demonstrates allele-specific complementation for  $\lambda$  and T4 of GroEL44 (Glu191→Gly) mutant (Tables 3 & 4). The effect is nevertheless incomplete; plaques on SV2 [pJCGroES] are invariably smaller than on SV1, or SV1

[pJCGroES]. The E191G single mutation blocks the assembly of the head structure of bacteriophage  $\lambda$  (14, 30). The substitution of glycine in the hinge region between the intermediate and apical domains of GroEL (9) presumably increases the flexibility of the hinge. The pivoting of the hinge region ensures proper interaction with GroES (47). For  
5 example, the mutant GroEL59 (Ser201→Phe in the same hinge region) in SV3 has low affinity for GroES (48). Over-expression of GroES will favour the formation of GroES-EL44 complex; we indeed also observed complementation of SV2 for thermosensitivity and bacteriophages growth by over-expressing GroEL44 mutant (data not shown). Taking  
10 advantage of the GroES effect, we observed that GroEL minichaperones and MC<sub>7</sub> all reduce both plaque size and number but, like GroEL, do not completely eliminate them in SV2 [pBADGroES].

**Table 3.** Growth of bacteriophage  $\lambda$  at 30 °C in transformed wild-type and *groEL* mutant strains.

Plasmids pJC	<i>groE</i> strains			
	SV1 ( <i>groEL</i> <sup>+</sup> )	SV2 <i>groEL44</i>	SV3 <i>GroEL59</i>	SV6 <i>groEL673</i>
short his tag (sht)	+++	-	-	-
( <i>ES</i> <sup>-</sup> , <i>EL</i> <sup>-</sup> )				
GroES (1-97)	+++	+++	-	-
Gp31 (1-111)	+++	-	-	-
Gp31?loop	+++	-	-	-
GroEL (1-548)	-	+	++	-
sht-GroEL (191-376)	+++	-	-	-
Gp31?::	+	-	-	-
GroEL (191-376)				

5    +++, normal plaque-forming ability relative to wild-type *groEL*<sup>+</sup> strain, in terms of both number and size; ++, 5-fold fewer plaques relative to wild-type *groEL*<sup>+</sup> strain, or both; +, 10-fold fewer plaques, or plaque size reduced relative to wild-type *groEL*<sup>+</sup> strain, or both; +/-, 10<sup>2</sup>-fold fewer plaques and plaque size reduced relative to wild-type *groEL*<sup>+</sup> strain; -, no visible plaques (<10<sup>-4</sup>).

**Table 4.** Growth of bacteriophage T4 at 37 °C in transformed wild-type and *groEL* mutant strains.

Plasmids pJC	<i>groE</i> strains			
	SV1 ( <i>groEL</i> <sup>+</sup> )	SV2 <i>groEL44</i>	SV3 <i>groEL59</i>	SV6 <i>groEL673</i>
short his tag (sht)	+++	-	+++	-
( <i>ES</i> <sup>-</sup> , <i>EL</i> <sup>-</sup> )				
GroES (1-97)	+++	+++	+++	-
Gp31 (1-111)	+++	+++	+++	+++
Gp31?loop	+/-	-	-	-
GroEL (1-548)	+++	+++	+++	+++
sht-GroEL (191-376)	+++	-	+++	-
Gp31?::	++	+	+	+/-
GroEL (191-376)				

5 +++ , normal plaque-forming ability relative to wild-type *groEL*<sup>+</sup> strain, in terms of both number and size; ++, 5-fold fewer plaques relative to wild-type *groEL*<sup>+</sup> strain, or both; +, 10-fold fewer plaques, or plaque size reduced relative to wild-type *groEL*<sup>+</sup> strain, or both; +/-, 10<sup>2</sup>-fold fewer plaques and plaque size reduced relative to wild-type *groEL*<sup>+</sup> strain; -, no visible plaques (<10<sup>-4</sup>).

## Claims.

- 5 1. A polypeptide monomer comprising an amino acid sequence inserted into the structure of a subunit of a heterologous oligomerisable scaffold.
2. A polypeptide according to claim 1, wherein the oligomerisable scaffold is bacteriophage T4 Gp31, and the amino acid sequence is inserted so as to substantially  
10 replace the mobile loop between positions 27 and 42.
3. A polypeptide oligomer comprising monomers according to claim 1 or claim 2.
4. A polypeptide oligomer according to claim 3, which is a homooligomer.
- 15 5. A polypeptide according to any preceding claim wherein the heterologous amino acid sequence is a minichaperone polypeptide.
6. A polypeptide comprising two or more minichaperone polypeptides covalently  
20 linked together.
7. A polypeptide according to claim 6, wherein the minichaperone polypeptides are arranged in a ring formation.
- 25 8. A polypeptide according to claim 6 or claim 7, which comprises seven minichaperone polypeptides.
9. A polypeptide according to any one of claims 5 to 8, wherein the minichaperone is a fragment of *E. coli* GroEL selected from the group consisting of residues 191-376, 191-  
30 345 and 193-335 of intact GroEL.

10. A polypeptide according to claim 9, wherein fragment of GroEL does not have an Alanine residue at position 262 and/or an Isoleucine residue at position 267 of the sequence of intact GroEL.
- 5 11. A polypeptide according to claim 10, wherein the fragment of GroEL has a Leucine residue at position 262 and/or a Methionine residue at position 267 of the sequence of intact GroEL.
12. A polypeptide according to any one of claims 5 to 11 for use in promoting the  
10 folding of an unfolded or misfolded polypeptide.
13. A method for promoting the folding of a polypeptide comprising contacting the polypeptide with a polypeptide according to any one of claims 5 to 11 and a foldase.
- 15 14. A method according to claim 13, wherein the polypeptide is an unfolded or misfolded polypeptide.
15. A method according to claim 14, wherein the polypeptide comprises a disulphide.
- 20 16. A method according to any preceding claim, wherein the foldase is selected from the group consisting of thiol/disulphide oxidoreductases and peptidyl-prolyl isomerases.
15. A method according to claim 14, wherein the thiol/disulphide oxidoreductase is selected from the group consisting of *E. coli* DsbA and mammalian PDI, or a derivative  
25 thereof.
16. A method according to claim 14, wherein the peptidyl prolyl isomerase is selected from the group consisting of cyclophilin, parbullen, SurA and FK506 binding proteins.
- 30 17. A method according to any one of claims 13 to 16, comprising contacting the polypeptide with a polypeptide according to any one of claims 5 to 11 and both a thiol/disulphide oxidoreductase and peptidyl-prolyl isomerase.



18. A method according to any one of claims 13 to 17, wherein the polypeptide according to any one of claims 5 to 11 and/or the foldase is immobilised onto a solid phase support.

5

19. A method according to claim 18 wherein the solid phase support is agarose.

20. A solid phase support having immobilised thereon a polypeptide according to any one of claims 5 to 11 and/or a foldase.

10

21. A column packed at least in part with a solid phase support according to claim 20.

22. Use of a polypeptide according to any one of claims 5 to 11 and a foldase for promoting the folding of a polypeptide.

15

23. Use according to claim 22 wherein the polypeptide according to any one of claims 5 to 11 and/or the foldase is immobilised on a solid phase support.

24. A composition comprising a combination of a polypeptide according to any one of claims 5 to 11 and a foldase.

20

**Abstract**

The invention provides a method for constructing polypeptide oligomers which are based on an oligomerisable scaffold subunit, to which the polypeptides are fused. Moreover, the  
5 invention provides minichaperone oligomeric peptides which are useful in promoting polypeptide folding.

Figure 1

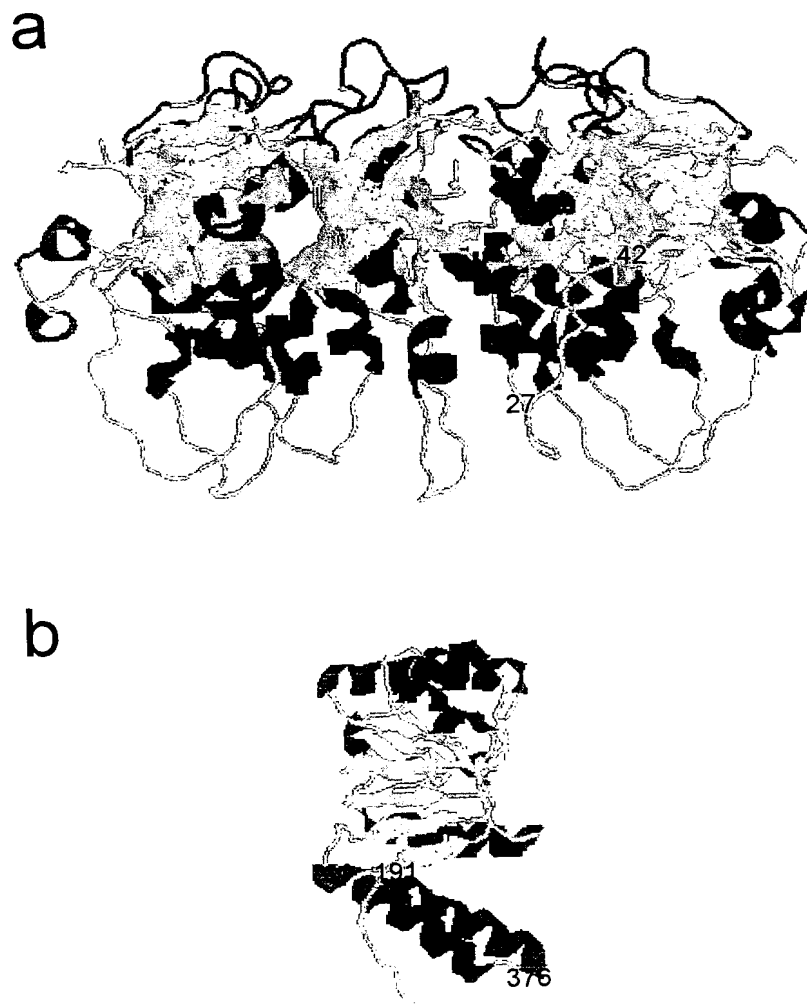




Figure 2

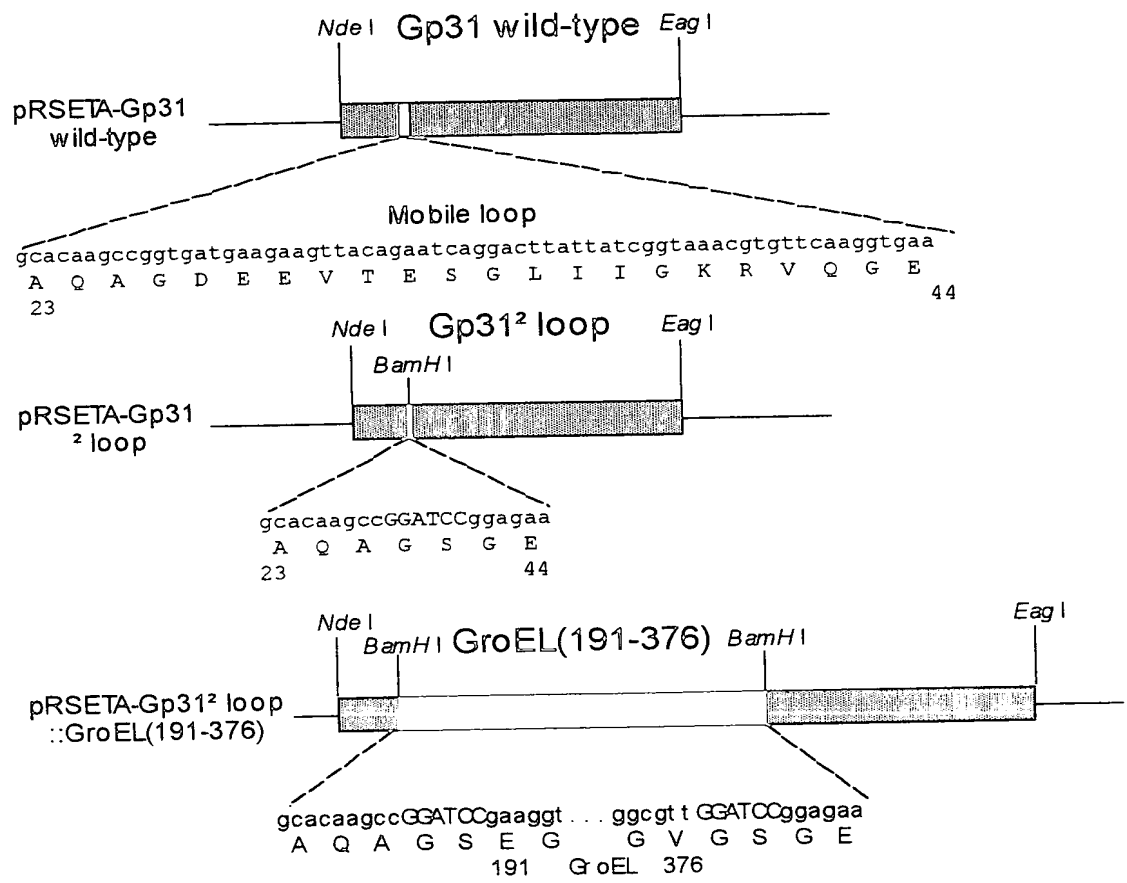




Figure3

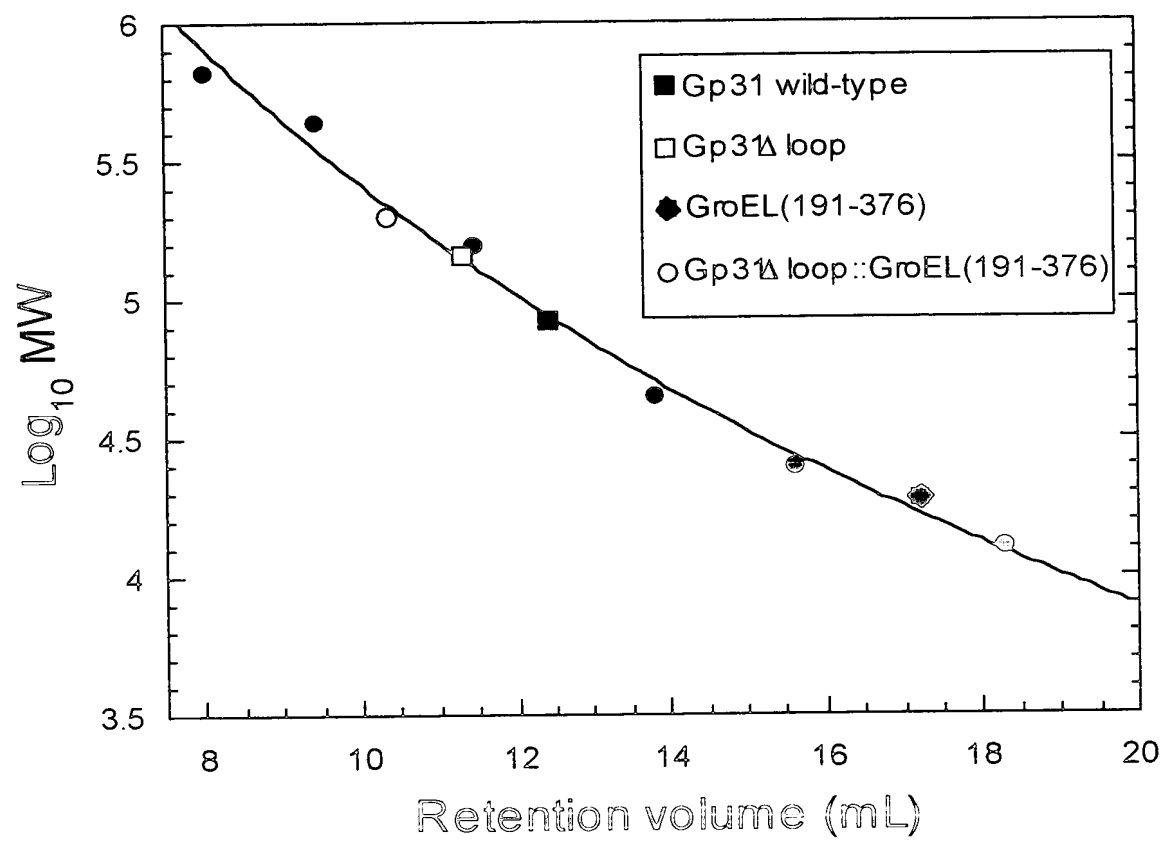






Figure 4

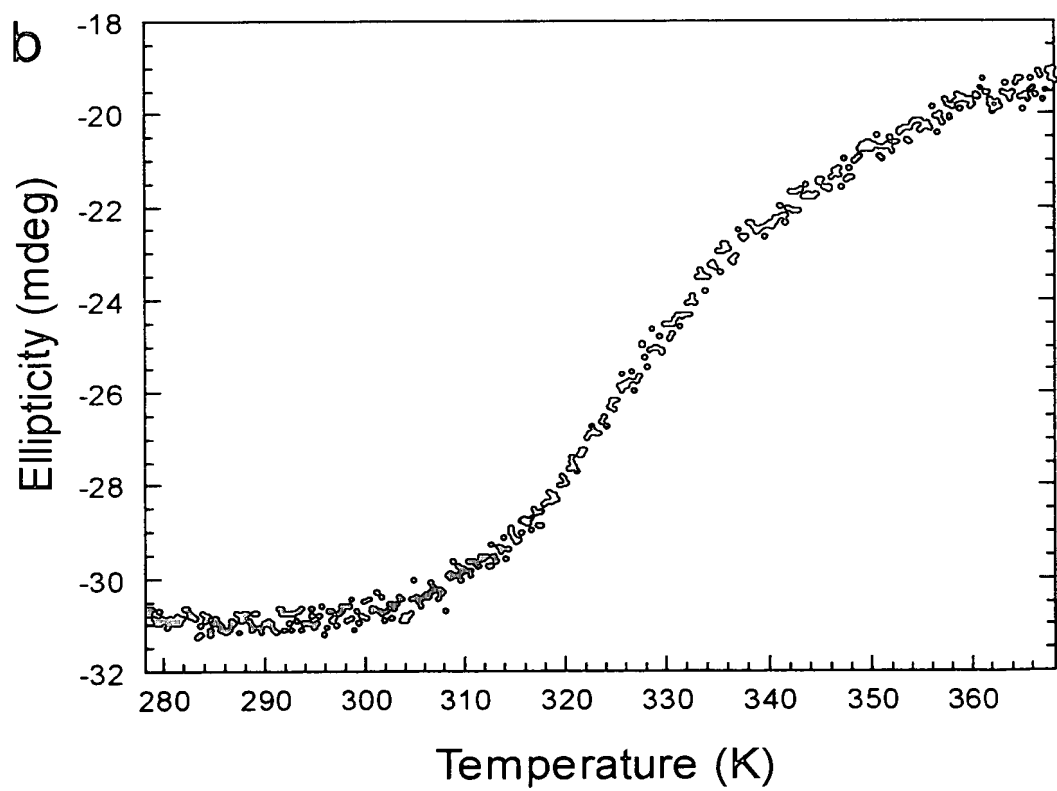
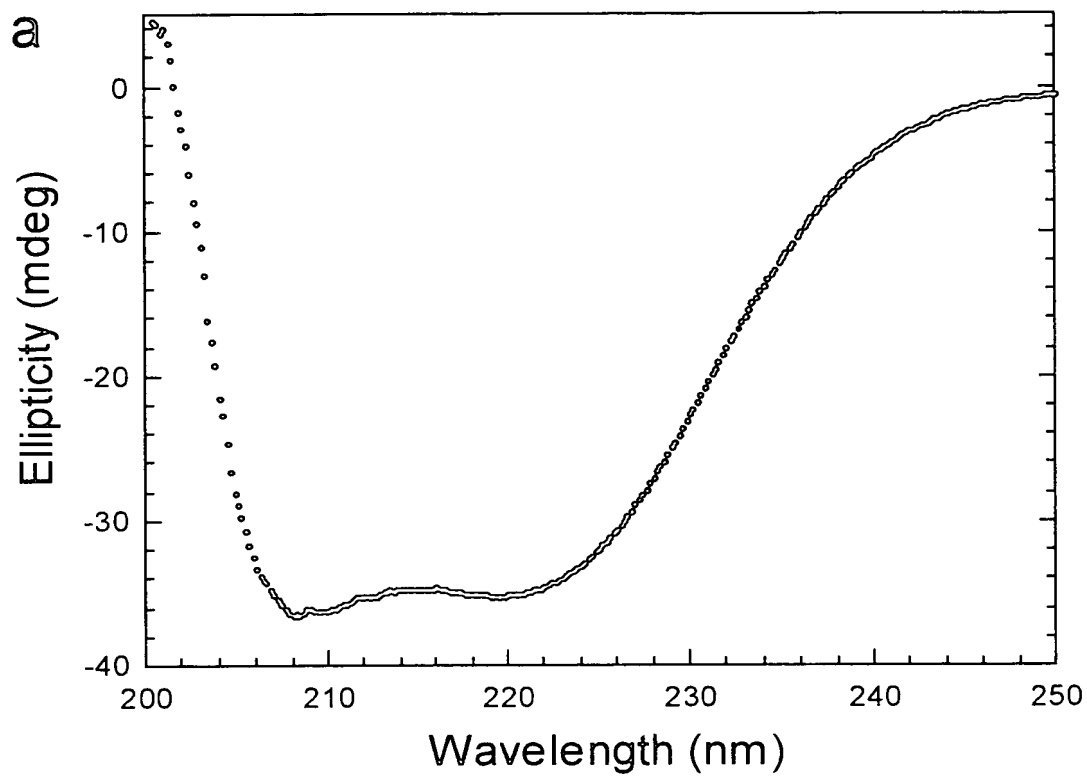




Figure 5

